

Anti-inflammatory role of microRNA-429 in human gingival epithelial cells-inhibition of IL-8 production through direct binding to IKK β mRNA

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Abstract. MicroRNAs (miRNAs), a family of small non-coding RNAs, serve a pivotal role in the regulation of the inflammation by modulating the expression of various genes. However, the molecular mechanism by which miRNAs regulate inflammation-associated molecules in oral epithelial cells remains to be elucidated. The present study examined the biological function of miR-429 by performing the gain-/loss-of-function studies of miR-429 in a gingival squamous cell carcinoma line Ca9-22 cells that either over- or under-expressed miR-429 through transient transfection with miR-429 mimic or miR-429 inhibitor, respectively. The results demonstrated that the over-expression of miR-429 suppressed the mRNA level of several interleukins, including IL-8. In addition, the over-expression of miR-429 reduced IL-8 secretion under the basal and TNF- α stimulated conditions, whereas the secretion of IL-8 was enhanced when miR-429 was under-expressed. The over-expression of miR-429 inhibited the activation of the transcription factor NF- κ B. Furthermore, we found that miR-429 suppressed both mRNA and protein levels of IKK β via its direct binding to the 3'-untranslated region of IKK β mRNA. In addition, the downregulation of IKK β by small interfering RNA reduced both NF- κ B activity and IL-8 production in Ca9-22 cells. Taken together, the findings revealed the molecular mechanism of miR-429 to regulate the inflammatory mediator in gingival cells and suggested that it could be useful as a therapeutic target of oral inflammatory diseases.

Introduction

Periodontitis, which is a representative inflammatory disease in the gingival tissues, is generally triggered by various

inflammation-induced factors derived from bacteria and host cells. If left untreated, the disease eventually causes tissue breakdown and alveolar bone loss. In addition, it may increase a risk of developing serious systemic complications such as diabetes (1-3). Inflammation is a protective immune response to injury and infection and responsible for maintaining homeostasis (1). However, excess and prolonged inflammation can lead to cellular damage and organ dysfunction (1,3). There are numerous molecules that can affect the progression of periodontal inflammation. Among them, TNF- α and interleukins such as IL-1, IL-6 and IL-8 are the central modulator of immune response and critically involved in inflammatory events (4,5). These molecules can activate the transcription factor NF- κ B and this activation occurs through phosphorylation of p65 and translocation of the p65/p50 dimer from the cytoplasm into the nuclei. Since interleukins and TNF- α are the targets of NF- κ B, this process results in a positive feedback loop promoting further inflammation (6). Thus, the precise regulation of this inflammatory signaling pathway is important to control and treat a number of inflammatory disorders. At present, however, preventive and therapeutic means for inflammatory oral diseases still need to be explored.

A microRNA (miRNA) is a small non-coding RNA that modulates gene expression at the post-transcriptional level via binding to the complementary sequences in 3'-untranslated region (3'-UTR) of target mRNAs (7). Growing evidence has suggested that miRNAs are involved in the regulation of diverse physiological and pathological events, such as cell differentiation, development, migration and apoptosis. A number of studies have demonstrated that miRNAs are often dysregulated and closely associated with solid and blood cell cancer and infectious inflammatory diseases (7-13). Furthermore, circulating extracellular miRNAs are stable and detectable in body fluid specimens from patients and their contents in the specimens may reflect or be attributed to a certain disease status (7-9,13-15). On the basis of these findings, miRNAs have emerged as an attractive and powerful tool for diagnostic biomarkers and therapeutic targets.

Among miRNAs, miR-429, a member of miR-200 family, is studied as an epithelial mesenchymal transition-associated miRNA and is often found to be dysregulated in various types

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of malignant tumors including oral squamous cell carcinoma (16-19). Previous studies have reported that miR-429 and its family miRNAs suppress the expression of multiple genes involved in inflammatory signaling and production of interleukins, particularly IL-8 (16-18,20-27). The evidence for the relevance and function of miR-429 in pathological and physiological events has accumulated; however, the possible association of miR-429 with oral inflammatory processes remains to be elucidated.

The present study aimed to investigate the molecular mechanisms of miR-429 action to suppress inflammation in oral mucosa using a human gingival squamous cell carcinoma line (Ca9-22). The results demonstrated that miR-429 inhibited IL-8 production through the NF- κ B pathway. In addition, miR-429 directly interacted with the IKK β , an essential activator of NF- κ B in the canonical pathway. Collectively, the results suggested that miR-429 has anti-inflammatory function in gingival epithelium, providing new information concerning with the role of miRNAs as a modulator of inflammatory cascade in oral diseases.

Materials and methods

Cell culture. Ca9-22 (JCRB0625, Lot. 11182016, Biomedical Innovation, Health and Nutrition Research Institute, authenticated by Japanese Collection of Research Bioresources Cell Bank), a human gingival squamous cell carcinoma line, was cultured in Dulbecco's modified Eagle's medium (DMEM) (cat. no. D5796; Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (cat. no. 10270; Gibco; Thermo Fisher Scientific, Inc.), 100,000 U/ml penicillin and 100 μ g/ml streptomycin (cat. no. 164-25251, Wako Pure Chemical Industries, Ltd.) in a humidified atmosphere of 5% CO₂ at 37°C.

Transfection. The Ca9-22 cells were seeded onto a 24-well plate (5x10⁴ cells per well) and onto a 12-well plate (1x10⁵ cells per well), respectively, for the evaluation of mRNA and protein levels. After the incubation for 24 h at 37°C, the cells were transfected with miR-429 mimic (sense, 5'-GGUUUU ACCAGACAGUAUATT-3' and antisense, 5'-UAAUAC UGUCUGGUA AACCGU-3'; miRVana miRNA mimic; assay ID MC10221; Thermo Fisher Scientific, Inc.), negative control mimic (NCm; miRVana miRNA mimic negative control #1; cat. no. 4464058; Thermo Fisher Scientific, Inc.), miR-429 inhibitor (5'-ACGGUUUU ACCAGACAGU AUUA-3'; miRVana miRNA inhibitor; assay ID MH10221; Thermo Fisher Scientific, Inc.), negative control inhibitor (NCi; miRVana miRNA Inhibitor Negative Control #1; cat. no. 4464076; Thermo Fisher Scientific, Inc.), small interfering (si) RNA targeting IKK β (siRNA-IKK β ; sense, 5'-GGG CAGUCUUUGCACUACATT-3' and antisense, 5'-UGA UGUGCAAAGACUGCCCTG-3'; Silencer select validated siRNA; assay ID s7263; Thermo Fisher Scientific, Inc.) or negative control siRNA (NCsi; Silencer select negative control No. #1; cat. no. 4390843; Thermo Fisher Scientific, Inc.) using Lipofectamine[®] RNAiMax (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols, and incubated for 24 or 48 h at 37°C. Then, the transfected cells were used for further experiments. The concentrations

of miR-429 mimic, NCm, miR-429 inhibitor and NCi were 50 nM and those of siRNA-IKK β and NCsi were 30 nM.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from cells stimulated with TNF- α (5, 10 or 50 ng/ml; cat. no. 207-15261; Wako Pure Chemical Industries, Ltd.) or the transfected cells using a Direct-zol miniprep RNA kit (cat. no. R2063; Zymo Research Corp.) with TRIzol[®] (Invitrogen; Thermo Fisher Scientific, Inc.). Conversion of total RNA into complementary DNA (cDNA) was performed by using a TaqMan miRNA reverse transcription kit (Thermo Fisher Scientific, Inc.) for miR-429 and U6 and by using a PrimeScript RT reagent kit with genomic DNA Eraser (cat. no. RR047A; Takara Bio, Inc.) for IL-1 β , IL-6, IL-8, IKK β and GAPDH. Both kits were used according to the manufacturers' protocols. qPCR was performed using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.) and the relative expression level was normalized with U6 or GAPDH expression and calculated based on the Cq ($\Delta\Delta C_q$) method (28). The expression level of miR-429 and U6 was examined according to the previous RT-qPCR condition (12), using TaqMan Fast universal PCR master mix (Thermo Fisher Scientific, Inc.) with TaqMan miRNA primers of miR-429 (assay ID 001024; Thermo Fisher Scientific, Inc.) and U6 (assay ID 001973; Thermo Fisher Scientific, Inc.). As reported previously (29), the mRNA level of IL-1 β , IL-6, IL-8, IKK β and GAPDH was examined according to the PCR reaction condition as follows: 30 sec at 95°C and subsequent 40 repeats of 10 sec at 95°C, 10 sec at 63°C and 15 sec at 72°C. The reaction was conducted using a SYBR Premix Ex Taq II (cat. no. RR820A; Takara Bio, Inc.) with specific primers as follows: Human IL-1 β (forward, 5'-AAACAGATGAAGTGC TCCTCC-3' and reverse, 5'-AAGATGAAGGGAAAGAAG GTGC-3'), human IL-6 (forward, 5'-AATCATCACTGG TCTTTTGGAG-3' and reverse, 5'-GCATTTGTGGTTGGG TCA-3'), human IL-8 (forward, 5'-GACATACTCCAAACC TTTCCACC-3' and reverse, 5'-AACTTCTCCACAACCCTC TGC-3'), human IKK β (forward, 5'-ACTTGGCGCCCAATG ACCT-3' and reverse, 5'-CTCTGTTCTCCTTGCTGCA-3') and GAPDH (forward, 5'-CTCATGACCACAGTCCATGC-3' and reverse, 5'-TTCAGCTCTGGGATGACCTT-3').

ELISA. The transfected Ca9-22 cells were incubated for 4 h at 37°C in the fresh culture medium with or without human recombinant TNF- α (50 ng/ml). Then, the medium was collected and stored at -20°C until use. The amount of IL-8 in the medium was measured with a human IL-8 Uncoated enzyme-linked immunosorbent assay kit (cat. no. 88-8086-22, Thermo Fisher Scientific, Inc.).

Western blotting. For evaluation of phosphorylated (p)-p65 and p65 levels, the transfected Ca9-22 cells were cultured in the fresh medium for 6 h at 37°C and then incubated in the medium with or without TNF- α (50 ng/ml) for 15 min at 37°C prior to protein extraction. Total protein of the transfected cells was extracted using RIPA lysis buffer (cat. no. 20-188; EMD Millipore) containing Halt protease and phosphatase inhibitor (cat. no. 1861280, Thermo Fisher Scientific, Inc.). The protein extract (10 μ g) was separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and

blotted onto a PVDF membrane. The membrane was blocked with 5% non-fat dried milk (cat. no. 999S; Cell Signaling Technology, Inc.) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Then, the membrane was incubated with the rabbit anti-IKK β (cat. no. 2678P; Cell Signaling Technology, Inc.), anti-p-NF- κ B (cat. no. 3033S; Cell Signaling Technology, Inc.) or anti-NF- κ B (cat. no. sc-109X; Santa Cruz Biotechnology, Inc.) in Can Get Signal Immunoreaction Enhancer Solution 1 (cat. no. NKB-201; Toyobo Life Science) at a 1:3,000 dilution overnight at 4°C, followed by the incubation with HRP-conjugated anti-rabbit IgG antibody (cat. no. 7074S; Cell Signaling Technology, Inc.) diluted 3,000 times in Can Get Signal Immunoreaction Enhancer Solution 2 (cat. no. NKB-301; Toyobo Life Science) for 1 h at room temperature. For the determination of GAPDH level, the membrane was incubated with the HRP-conjugated anti-GAPDH antibody (cat. no. 015-25473, Wako Pure Chemical Industries, Ltd.) in TBS-T buffer containing 5% non-fat dry milk at a 1:10,000 dilution for 1 h at room temperature. GAPDH was used for normalization of phosphorylated p65 (p-p65), p65 and IKK β and the p-p65 level was normalized relative to the p65 level. The immunoreactive proteins were detected with Immobilon Forte Western HRP Substrate (cat. no. WBLUF0100; EMD Millipore) and the bands were visualized and analyzed on a V3 Western Workflow (Bio-Rad Laboratories, Inc.) using Image Lab software (version 6.0.1; Bio-Rad Laboratories, Inc.).

In silico miRNA target gene prediction. TargetScan (version 7.1; <http://www.targetscan.org/>) and DIANA-microT-CDS (version 5.0; http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) were used to predict the putative target genes of miR-429.

Dual luciferase assay. For the measurement of NF- κ B activity, a plasmid possessing five tandem repeats of NF- κ B transcription responsive elements (TGGGGACTTTCCGC) inserted into pGL3-promoter vector (cat. no. E1761; Promega Corporation) was constructed, as previously reported (30). The Ca9-22 cells were seeded onto a 48-well plate at 2.5×10^4 cells per well and incubated for 24 h at 37°C. The plasmid (100 ng) was co-transfected with pRL *Renilla* luciferase reporter vector (50 ng; cat. no. E2241; Promega Corporation) and miR-429 mimic, miR-429 inhibitor or the corresponding NC using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After co-transfection for 24 h, the cells were cultured in fresh medium overnight and then treated with or without TNF- α (50 ng/ml) for 6 h at 37°C. The firefly and *Renilla* luciferase activities were measured using a Dual Luciferase Reporter Assay kit (cat. no. E1910; Promega Corporation). The firefly luciferase activity was normalized to *Renilla* luciferase activity. To determine whether miR-429 binds to its potential target, a plasmid with IKK β 3'-UTR wild type (WT; IKK β -WT) was prepared by cloning the 3'-UTR of IKK β into pmirGLO plasmid (cat. no. E1330; Promega Corporation). A plasmid with IKK β 3'-UTR mutant type (MUT; IKK β -MUT), which has five mutated nucleotides (5'-CAGTATT) in the predicted target site of miR-429 in 3'-UTR of IKK β (5'-GACATTA), was prepared by using inverse PCR with

In-Fusion HD Cloning kit (cat. no. 639648; Takara Bio, Inc.) with IKK β -WT as a template. The Ca9-22 cells were seeded onto a 48-well plate at 2.5×10^4 cells per well and incubated for 24 h at 37°C. Then, either WT or MUT luciferase plasmid (200 ng) was co-transfected with miR-429 mimic, miR-429 inhibitor or the corresponding NC using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The luciferase assay was performed as described above.

Statistical analysis. The data were expressed as the mean \pm standard deviation (SD) of three or four independent experiments. Statistical differences were determined using Student's t-test, one-way or two-way ANOVA followed by Bonferroni's multiple comparison test. Statistical analyses were performed using WinSTAT (R. Fitch Software). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-429 downregulates IL-8 mRNA level and inhibits its secretion in gingival epithelial cells. To evaluate the biological function of miR-429 in oral inflammation, Ca9-22 cells with over- and under-expressed miR-429 were prepared through transient transfection with chemically modified oligonucleotides, miR-429 mimic and miR-429 inhibitor, respectively. As a control, Ca9-22 cells transfected with modified oligonucleotides having scramble sequences, NCm for miR-429 mimic and NCi for miR-429 inhibitor, were used. Successful over- and under-expressions of miR-429 in the cells were confirmed by the analysis using RT-qPCR (Fig. 1A and B). In addition, the change in the level of miR-429 in inflammatory conditions were examined. Under TNF- α stimulation, there was a tendency of decrease in miR-429 expression in Ca9-22 cells (Fig. 1C). Next, the effect of miR-429 on the mRNA level of IL-1 β , IL-6 and IL-8 was evaluated using the transfected cells. As shown in Fig. 2A-C, the expression of IL-1 β and IL-8 mRNAs was significantly decreased by the over-expression of miR-429, whereas the IL-6 mRNA level was not changed significantly. By contrast, the under-expression of miR-429 significantly increased the mRNA level of IL-1 β and IL-8 but not that of IL-6 (Fig. 2D-F). These results indicated that miR-429 inhibited the expression of IL-1 β and IL-8 at a transcriptional level.

Next, the concentration of IL-8 in the conditional medium of Ca9-22 cells was measured by ELISA (Fig. 3A and B). Under the basal condition, the miR-429 mimic significantly reduced IL-8 secretion from the cells whereas the miR-429 inhibitor significantly increased the secretion when compared to the corresponding NC group. Similarly, under TNF- α stimulated condition where the IL-8 secretion was significantly increased, the miR-429 mimic significantly inhibited the IL-8 secretion whereas the miR-429 inhibitor further enhanced the secretion when compared to the cells treated with NCm or NCi. In addition, the IL-1 β in the conditional medium was measured, but it was undetectable under the conditions used in the present study (data not shown). Taken together, the above results suggested that miR-429 exerts anti-inflammatory action through the suppression of IL-8 production in Ca9-22 cells.

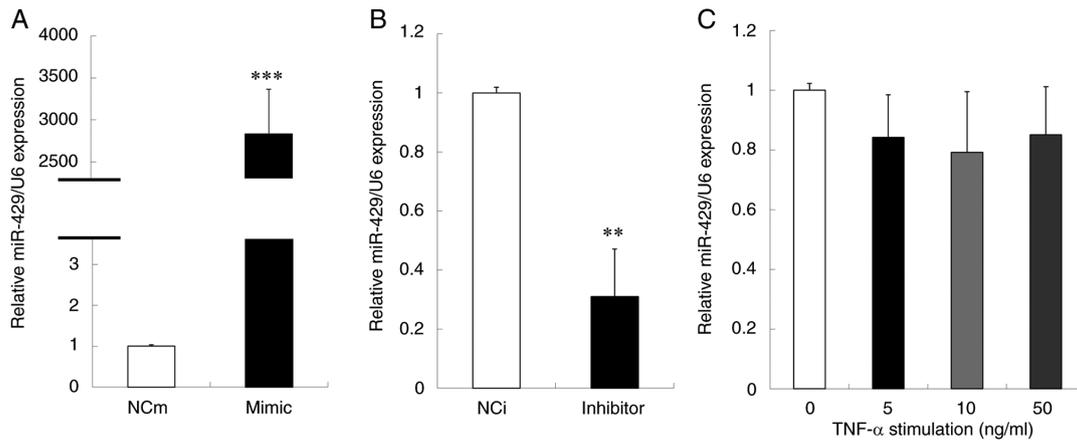


Figure 1. Over- and under-expression of miR-429 in Ca9-22 cells. Ca9-22 cells were transfected with (A) miR-429 mimic or NCM and with (B) miR-429 inhibitor or NCI or (C) stimulated with TNF- α (5, 10 or 50 ng/ml). The expression level of miR-429 was determined by reverse transcription-quantitative PCR. U6 was used as a housekeeping gene control. Data are presented as the means \pm standard deviation (n=3). The t-test or one-way ANOVA followed by Bonferroni's multiple comparison test was used for the calculation of P-values. **P<0.01, ***P<0.001 vs. the control group. miR, microRNA; mimic, miR-429 mimic; NCM, negative control mimic; inhibitor, miR-429 inhibitor; NCI, negative control inhibitor.

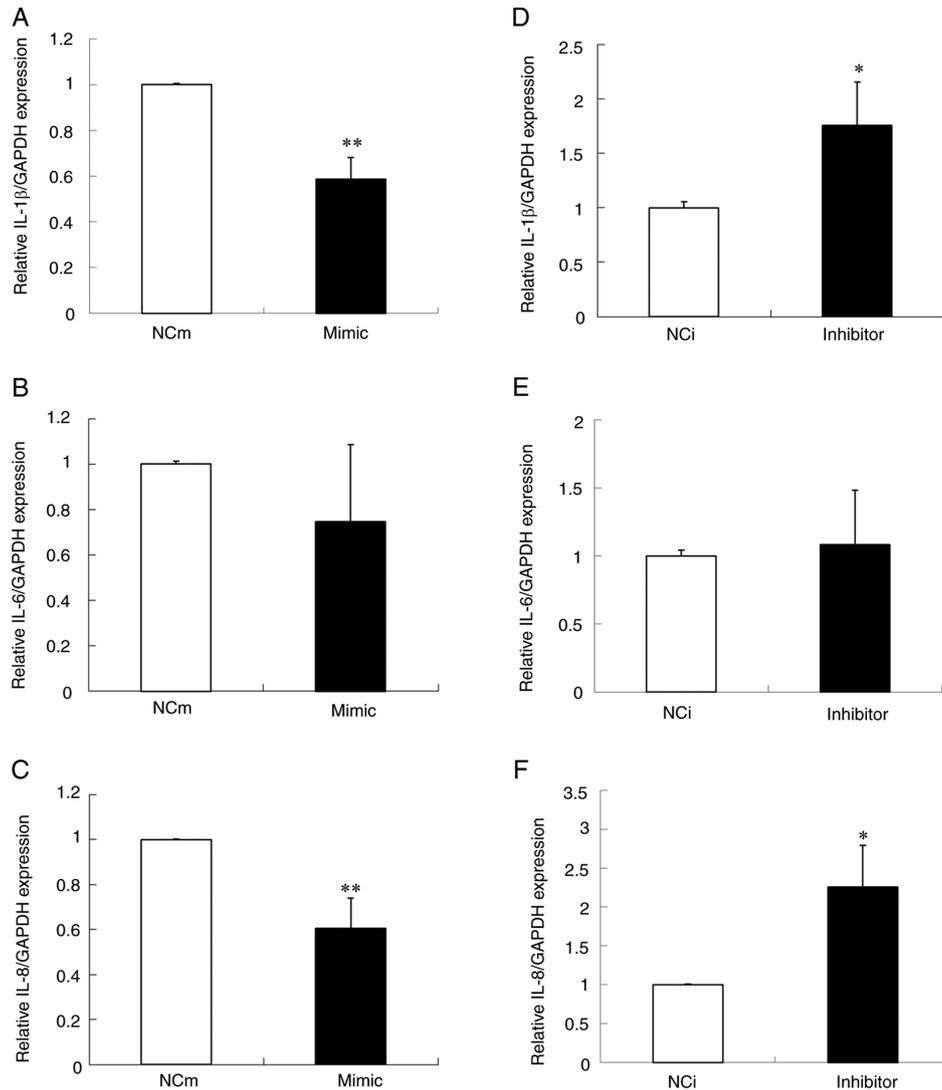


Figure 2. The effect of over- and under-expression of miR-429 on the expression of proinflammatory cytokines in Ca9-22 cells. Expression levels of proinflammatory cytokines, (A) IL-1 β , (B) IL-6 and (C) IL-8, in Ca9-22 cells transfected with miR-429 mimic or NCM were determined by reverse transcription-quantitative PCR. Expression levels of proinflammatory cytokines, (D) IL-1 β , (E) IL-6 and (F) IL-8, in Ca9-22 cells transfected with miR-429 inhibitor or NCI were also determined. GAPDH was used as a housekeeping gene control. Data are presented as the means \pm standard deviation (n=3). The t-test was used for the calculation of P-values. *P<0.05, **P<0.01 vs. the control group. miR, microRNA; mimic, miR-429 mimic; NCM, negative control mimic; inhibitor, miR-429 inhibitor; NCI, negative control inhibitor.

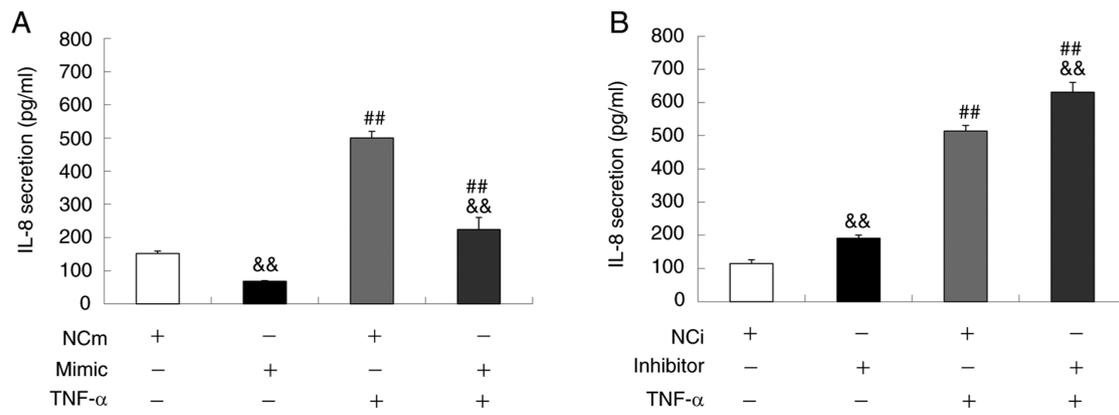


Figure 3. The effect of over- and under-expression of miR-429 on the secretion of IL-8 in Ca9-22 cells. Following the transfection of miR-429 mimic, NCm, miR-429 inhibitor or NCi, the cells were cultured with or without TNF- α (50 ng/ml). The concentration of IL-8 secreted in the medium was measured by ELISA. The amount of IL-8 derived from the cells transfected with the (A) miR-429 mimic or (B) miR-429 inhibitor was compared with that of the corresponding NC group. The IL-8 production was compared between the conditions with or without TNF- α stimulation under the same experimental conditions. Data are presented as the means \pm standard deviation (n=3). Two-way ANOVA followed by Bonferroni's multiple comparison test was used for the calculation of P-values. &&P<0.01 vs. the control group. ##P<0.01 vs. no TNF- α stimulation group. miR, microRNA; mimic, miR-429 mimic; NCm, negative control mimic; inhibitor, miR-429 inhibitor; NCi, negative control inhibitor.

miR-429 suppresses NF- κ B activation in gingival epithelial cells. Since miR-429 inhibited both mRNA expression and protein secretion of IL-8, the effect of miR-429 on the NF- κ B activation in Ca9-22 cells was next examined. The results demonstrated that over-expression of miR-429 significantly decreased the p-p65 level under both the basal and TNF- α treatment conditions (Fig. 4A). By contrast, the under-expression of miR-429 resulted in the significant increase of p-p65 level under those two conditions (Fig. 4B). To further confirm the effect of miR-429 on NF- κ B activity, a luciferase reporter plasmid possessing NF- κ B transcription responsive elements was constructed and the transcriptional activity of NF- κ B was evaluated by performing luciferase assay. The results demonstrated that the upregulation of miR-429 decreased the activity whereas its downregulation resulted in the opposite effect (Fig. 4C and D), indicating that miR-429 negatively regulates the transcriptional activity of NF- κ B.

miR-429 directly downregulates IKK β molecule in gingival epithelial cells. To gain a deeper understanding of miR-429 function in inflammatory process, experiments were performed to identify target genes of miR-429 in Ca9-22 cells. *In silico* prediction tools (TargetScan and DIANA-microT-CDS) were used to select the possible target genes of miR-429 and selected IKK β as a target candidate as it was predicted by both tools and is indeed one of the molecules in the NF- κ B pathway. As shown in Fig. 5A, the seed sequence of miR-429 has the complementary site in the 3'-UTR of the IKK β mRNA, indicating the direct interaction between miR-429 and IKK β . Thus, the effect of miR-429 on the expression of IKK β in Ca9-22 cells was examined. As shown in Fig. 5B and D, the over-expression of miR-429 significantly inhibited the mRNA and protein expressions of IKK β . By contrast, the under-expression of miR-429 demonstrated a tendency to increase the mRNA level of IKK β , but did not change its protein level (Fig. 5C and D). The present study further attempted to optimize the experimental conditions such as the concentration of miR-429 inhibitor (50 and 100 nM) and the treatment

time and re-examined the effect of miR-429 inhibitor on the level of IKK β protein. However, no significant changes in the protein level were found under all the conditions tested (data not shown).

To further examine whether miR-429 directly bound to 3'-UTR of IKK β , plasmid vectors that contain either WT 3'-UTR of IKK β (IKK β -WT) or MUT 3'-UTR of IKK β (IKK β -MUT) were constructed (Fig. 5A) and luciferase reporter assay was conducted. As shown in Fig. 5E and F, the luciferase activity of Ca9-22 cells transfected with IKK β -WT was significantly decreased by the co-transfection of miR-429 mimic, whereas co-transfection of miR-429 inhibitor did not affect the activity. In addition, either co-transfection of miR-429 mimic or miR-429 inhibitor did not alter the luciferase activity of the cells transfected with IKK β -MUT (Fig. 5E and F). Collectively, these results suggested that miR-429 directly bound to 3'-UTR of IKK β gene.

Downregulation of IKK β leads to the suppression of IL-8 production in gingival epithelial cells. Previous studies demonstrated that IKK β knockdown in some cells causes the attenuation of IL-8 expression and secretion (31-33). To examine whether the inhibitory effect of miR-429 on IL-8 secretion and p-p65 level was mediated via IKK β downregulation, Ca9-22 cells were transfected with siRNA targeting IKK β (siRNA-IKK β) or NCsi as a control and then the effect on the level of p-p65 and IL-8 secretion was analyzed. As shown in Fig. 6A and C, it was confirmed that the transfection with siRNA-IKK β inhibited the mRNA and protein levels of IKK β expression. In addition, the downregulation of IKK β expression was accompanied by the significant blockade of the phosphorylation of p65 under both the basal and TNF- α treatment conditions (Fig. 6D). Furthermore, it was found that the transfection with siRNA-IKK β significantly decreased the mRNA expression and protein secretion of IL-8 (Fig. 6B and E). These results indicated that the downregulation of IKK β in Ca9-22 cells resulted in the suppression of IL-8 production via inhibition of NF- κ B signaling.

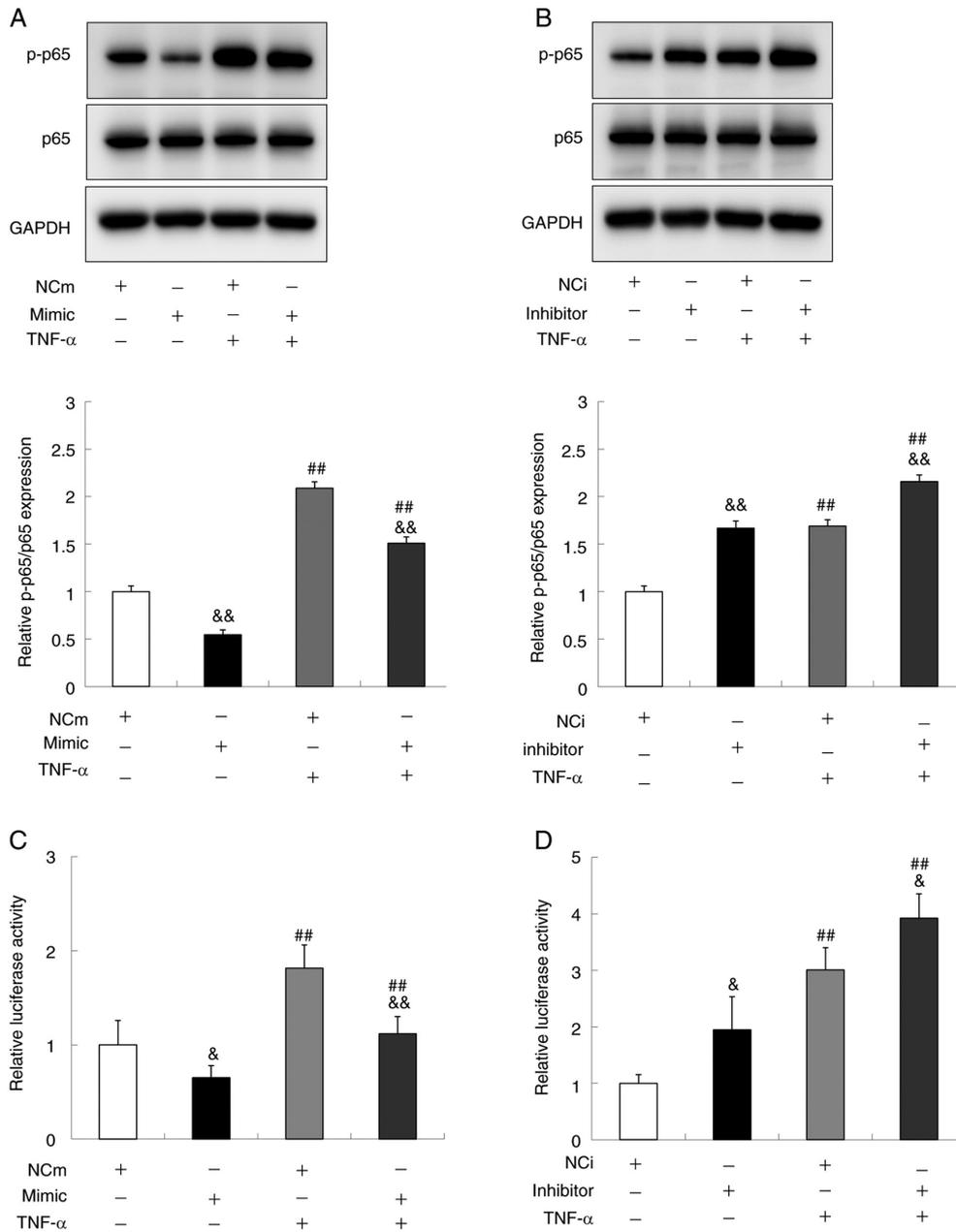


Figure 4. The effect of over- and under-expression of miR-429 on the activation of NF- κ B in Ca9-22 cells. The expression level of NF- κ B p65 protein was measured by Western blot analysis after the incubation of Ca9-22 cells, transfected with (A) miR-429 mimic or NCm and with (B) miR-429 inhibitor or NCI, with or without the subsequent stimulation of TNF- α (50 ng/ml). The levels of p-p65 and p65 were normalized by GAPDH level and the relative p-p65/p65 level was calculated. Data are presented as the means \pm standard deviation (n=3). The luciferase activity in the cells was determined by dual luciferase reporter assay system after the co-transfection of the plasmids with (C) miR-429 mimic or NCm and with (D) miR-429 inhibitor or NCI. Data are presented as the means \pm standard deviation (n=4). Two-way ANOVA followed by Bonferroni's multiple comparison test was used for the calculation of P-values. [&]P<0.05, ^{&&}P<0.01 vs. the control group. ^{##}P<0.01 vs. no TNF- α stimulation group. miR, microRNA; mimic, miR-429 mimic; NCm, negative control mimic; inhibitor, miR-429 inhibitor; NCI, negative control inhibitor; p-, phosphorylated.

Discussion

The oral mucosa is a unique tissue, which is constantly exposed to various types of endogenous and foreign substances including bacteria and other virulent factors (2,4,5,34). To protect the host from the pathogens, the immune system in mucosa initiates a series of inflammatory processes via immune cell activation, cell-cell communication and the release of pro-inflammatory mediators such as interleukins. Although inflammatory responses are essential for host defense against the pathogens, excess inflammation gives rise

to serious cellular damage, leading to tissue disruption and organ dysfunction. Hence, the precise control of inflammatory processes is necessary to maintain oral health; however, the molecular mechanism by which inflammation is regulated in the gingival mucosa remains to be fully elucidated.

The miR-200 family, including miR-429, has been studied for its biological function and aberrant expression in various diseases, particularly in tumors (16-27,35,36). Although several studies demonstrate that miR-429 exerted anti-inflammatory activities in some diseases (35,36), further investigation is necessary to fully understand its biological

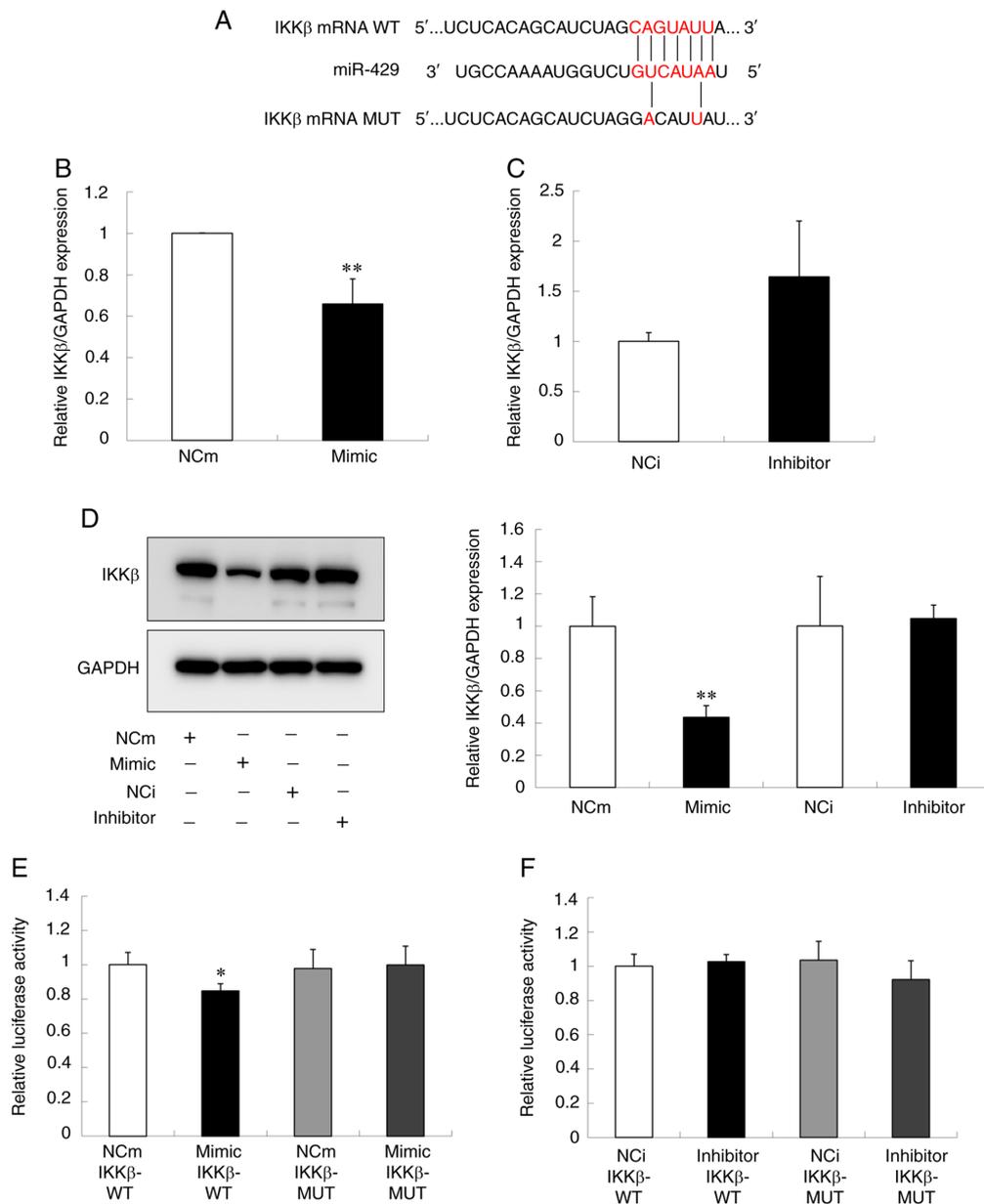


Figure 5. miR-429 direct binding to IKKβ in Ca9-22 cells. (A) The putative binding site of miR-429 on IKKβ-WT and its corresponding site on IKKβ-MUT. The cells were transfected with miR-429 mimic, miR-429 inhibitor or the corresponding NC. The levels of IKKβ (B and C) mRNA and (D) protein following the treatment with miR-429 mimic and with miR-429 inhibitor are shown. The IKKβ mRNA and protein levels were determined by reverse transcription-quantitative PCR and western blot analysis, respectively. GAPDH was used as a housekeeping gene control. (E and F) The luciferase assay in Ca9-22 cells co-transfected with IKKβ (IKKβ-WT or IKKβ-MUT) and (E) miR-429 mimic or NCm and (F) miR-429 inhibitor or NCI. The luciferase activity was determined by dual luciferase reporter assay system. Data are presented as the means ± standard deviation (n=3). The t-test was used for the calculation of P-values. *P<0.05, **P<0.01 vs. the control group. miR, microRNA; WT, wild type; MUT, mutant type; mimic, miR-429 mimic; NCm, negative control mimic; inhibitor, miR-429 inhibitor; NCI, negative control inhibitor.

and pathological roles in the inflammatory reaction in the oral mucosa. Therefore, the present study was performed to clarify the relationship between action of miR-429 and production of inflammatory mediator in gingival epithelial cells.

IL-8 is a potent inflammatory mediator and stimulates neutrophil and lymphocyte recruitment, tissue remodeling and angiogenesis (2,5). It is known that NF-κB activation triggers the production of IL-8 (37). A recent meta-analysis revealed that IL-8 level in inflamed gingival tissues is higher than that in the normal tissues (38), suggesting the involvement of IL-8 in oral inflammation. Accordingly, the present study examined the effect of miR-429 on the expression of IL-8 in Ca9-22

cells, a gingival squamous cell carcinoma line and found that the over-expression of miR-429 inhibited the IL-8 expression, whereas its enhanced expression was observed by the under-expression of miR-429 (Fig. 2C and F). Notably, several studies by other groups reported that some members of miR-200 family suppress the secretion of interleukins, especially IL-8 (20-24,26,35). These findings were confirmed by the present study. It was found that the over-expression of miR-429 attenuated IL-8 secretion in Ca9-22 cells regardless of TNF-α stimulation (Fig. 3), suggesting that miR-429 negatively regulated the production of IL-8 in oral mucosa. Next, the present study investigated the site of action of miR-429 to

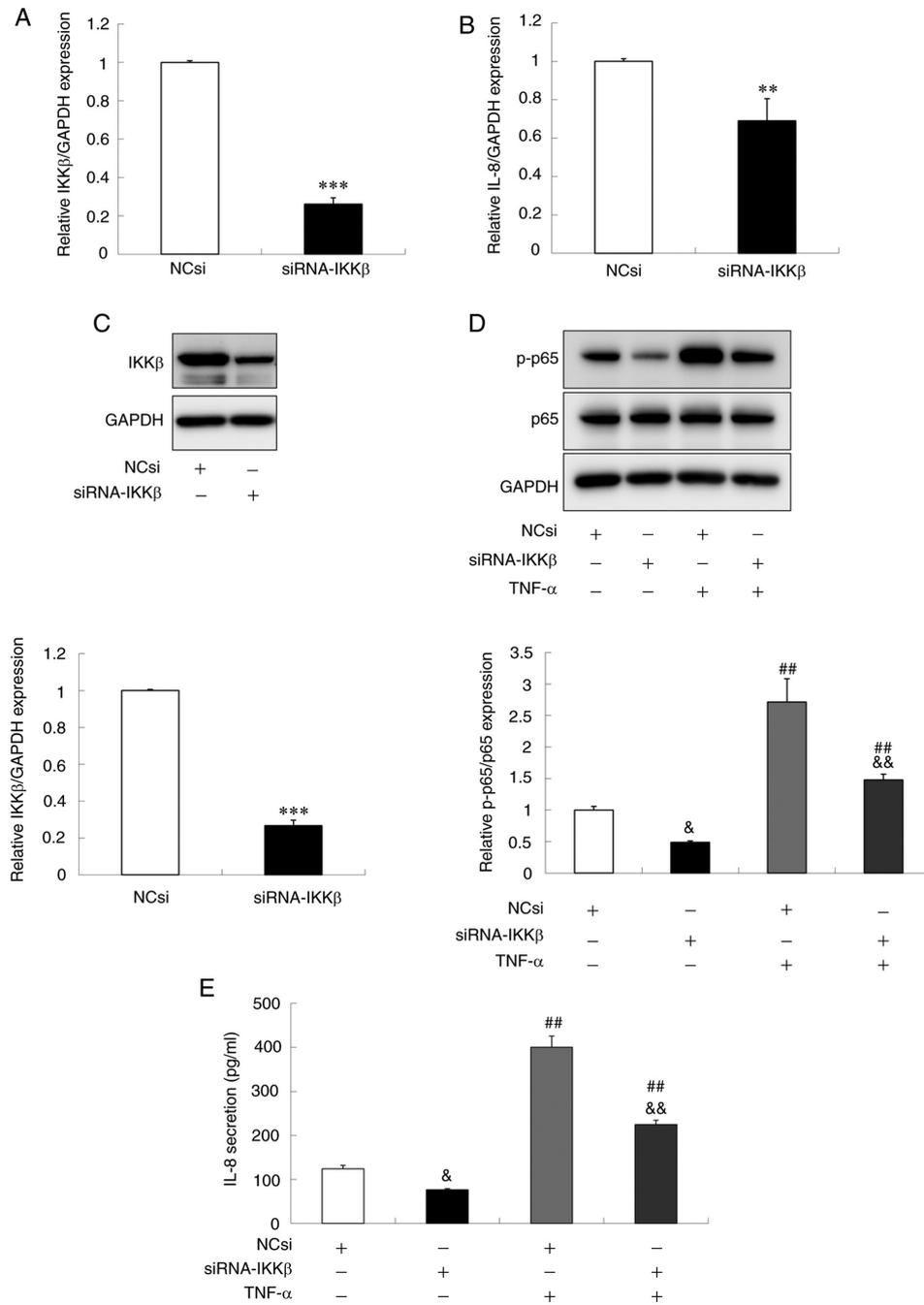


Figure 6. Effect of siRNA-IKKβ on IL-8 and NF-κB levels in Ca9-22 cells. The expression of (A) IKKβ and (B) IL-8 mRNA was analyzed by reverse transcription-quantitative PCR and GAPDH was used as a housekeeping gene control. (C) The expression of IKKβ protein. (D) The levels of p-p65 and p65 are shown after the treatment with siRNA-IKKβ or NCsi, with or without TNF-α stimulation (50 ng/ml). The protein level was measured by western blot analysis. The levels of IKKβ, p-p65 and p65 were normalized by GAPDH level and the relative p-p65/p65 level was calculated. (E) IL-8 secretion from Ca9-22 cells transfected with siRNA-IKKβ or NCsi, with or without TNF-α stimulation (50 ng/ml). The concentration of IL-8 secreted in the medium was measured by ELISA. Data are presented as the means ± standard deviation (n=3). **P<0.01, ***P<0.001 (Student's t-test), &P<0.05, &&P<0.01 (two-way ANOVA followed by Bonferroni's multiple comparison test) vs. the control group. ##P<0.01 (two-way ANOVA followed by Bonferroni's multiple comparison test) vs. the no TNF-α stimulation group. siRNA-IKKβ, small interfering RNA targeting IKKβ; p-, phosphorylated; NCsi, negative control siRNA.

inhibit IL-8 expression. It has been reported that miR-200c, a member of miR-200 family having the same seed sequence as miR-429, suppresses NF-κB binding activity in IL-8 promoter in leiomyoma smooth muscle cells, resulting in downregulation of IL-8 (20). The effect of miR-429 on the signaling pathway of NF-κB in Ca9-22 cells was examined and it was found that the over-expression of miR-429 suppressed NF-κB p65 phosphorylation, an indicator of NF-κB activation (Fig. 4).

IL-1β is also known as the inflammatory cytokine in the epithelial cells such as Ca9-22. The present study also demonstrated that miR-429 inhibited the IL-1β expression in Ca9-22 cells (Fig. 2A). However, it was not possible to examine the effect of miR-429 on the secretion of IL-1β protein from the cells, because IL-1β was not detectable under the condition used in this study (data not shown). Nagahama *et al* (39) examined the secretion of IL-8 and IL-1β from Ca9-22 cells and

found that the secreted amount of IL-8 was ~10-fold greater than that of IL-1 β . These data support the importance of IL-8 as a defense molecule in epithelial cells.

In this study, although the expression of miR-429 was enhanced over 2,000-fold (Fig. 1A), a relatively small effect on the mRNA level of IL-1 β (Fig. 2A) and IL-8 (Fig. 2C) was observed. Grimm *et al* (40) demonstrated that the high abundance of miRNA led to saturated Argonaute (AGO) 2 protein. A report by Matsui *et al* (41) demonstrated that the inhibition of AGO expression by siRNA reduced the silencing activity of the double-stranded miRNA mimic. These previous studies indicated the importance of AGO proteins in the mechanism of miRNA-mediated silencing. Based on these findings, we speculate that the effect of miR-429 mimic on the level of interleukins mRNA could be limited by the availability of AGO proteins.

IL-6 is another cytokine whose expression is regulated by NF- κ B; however, the level of IL-6 mRNA was not changed significantly in Ca9-22 cells by the treatment of miR-429 mimic and inhibitor (Fig. 2B and E) while miR-429 reduced the activity of NF- κ B (Fig. 4). It was hypothesized that the expression of IL-6 in carcinoma cell lines was also regulated by some other transcriptional factors as previously described (4,42,43). It is also possible that IL-6 expression may be regulated at the post transcriptional level affecting its stability (43).

The inhibitor of NF- κ B (I κ B) kinase (IKK) complex is composed of two catalytic subunits (IKK α and IKK β) and one regulatory subunit (IKK γ /NEMO) and phosphorylates I κ B that blocks the transcriptional activity of NF- κ B. Thus, this phosphorylation promotes the degradation of I κ B and thereby leads to NF- κ B activation (6). It was noteworthy that, unlike IKK α , IKK β is essential for inflammation-induced bone loss (44). In addition, a previous study demonstrates that oral bacteria and their products caused IKK β activation in oral epithelia cells, leading to inflammatory responses (45). Thus, IKK β is known as one of the key mediators of NF- κ B activation. The present study used *in silico* prediction tools (TargetScan and DIANA-microT-CDS) to identify a target gene of miR-429 in NF- κ B pathway and selected IKK β as a candidate. It was demonstrated that miR-429 suppressed IKK β at both the transcriptional and translational levels (Fig. 5B and D). In addition, miR-429 bound to 3'-UTR of IKK β in Ca9-22 cells, although the binding between miR-429 and IKK β 3'-UTR was weak according to the luciferase assay (Fig. 5E), which is a limitation of the present study. Some other means such as RNA immunoprecipitation assay could help strengthen this finding. In addition, the present study confirmed that the downregulation of IKK β by siRNA leads to the decreased production of IL-8 (Fig. 6). Taken together, it was demonstrated that miR-429 suppressed IL-8 production, at least in part, through inhibiting NF- κ B activation via direct binding to IKK β . It has been reported that some miRNAs including miR-16, miR-148a, miR-199a, miR-497 and miR-200 negatively regulate IKK β in a number of different types of cells (20,24,26,46-56). The present study provided the additional information that miR-429 is directly involved in the regulation of IKK β in gingival epithelial cells.

The present study was unable to demonstrate that the loss-of-function of miR-429 caused the significant increase of IKK β expression (Fig. 5C and D). This may be explained

by the insufficient inhibition of miR-429 in the protocol for transient transfection. Another possibility is that the translation of IKK β protein may be more complex and thus regulated by several other miRNAs as suggested in the previous studies (20,24,26,46-56). It could be that the inhibition of miR-429 alone may be insufficient to induce the upregulation of IKK β protein (57). Other experimental approaches for the deletion of miR-429 expression such as genome editing may help clarify this issue (58).

In conclusion, the present study found that miR-429 serves as an anti-inflammatory agent that inhibits the production of inflammatory cytokines such as IL-8 by inhibiting NF- κ B pathway via binding to IKK β of the IKK complex. To the best of our knowledge, this is the first evidence that miR-429 directly regulates IKK β in gingival epithelial cells and suggests that miR-429 could be a useful target for the treatment of inflammatory disorders in the gingival mucosa.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HK and HA designed the experiments, performed the data analysis and confirm the authenticity of all the raw data. HK performed the experiments and was a major contributor in writing the manuscript. HA revised the manuscript and gave the final approval of the version to be submitted. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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